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Minireview

Alternative Strategies for Becoming an Insider: Lessons from the Bacterial World

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Many microbial pathogens that have sustained long-standing associations with their vertebrate hosts have evolved sophisticated strategies to subvert cellular functions. These strategies are not accidental but rather the result of evolutionary forces operating over extended periods of time to secure the survival of both the pathogen and the host. Therefore, it should not come as a surprise that our developing understanding of pathogen/host cell interactions is not only uncovering wonderful biology but also teaching us useful lessons about the inner workings of the cell.

An essential step in the life cycle of many important pathogenic bacteria is their ability to invade cells that are normally nonphagocytic. Gaining access to an intracellular niche provides bacteria with an environment permissive for growth, allows them to avoid host defense mechanisms, or permits them to gain access to deeper tissues. Although certain cells such as macrophages are able to internalize particles of the size of a bacterium, gaining access into nonphagocytic cells requires very specific adaptations. The mechanisms by which some pathogenic bacteria enter into host cells is beginning to be understood in some detail. Although significantly different at first glance, there are clearly common themes among the different strategies utilized by different pathogenic bacteria to invade cells. Some bacteria have evolved determinants capable of engaging cell surface receptors to trigger signaling events that result in their internalization (Cossart and Lecuit, 1998; Isberg et al., 2000). Others have evolved strategies to engage the signaling machinery within the cell by "injecting" bacterial proteins that directly stimulate cellular events leading to bacterial uptake (Galan and Zhou, 2000). Both strategies ultimately involve the activation of signal transduction pathways to promote actin cytoskeleton rearrangements that drive bacterial internalization. A recent paper (Braun et al., 2000) and a paper published in this issue of Cell (Shen et al., 2000) have provided important insight into the mechanisms by which one of these bacteria, *Listeria monocytogenes*, enters into cells.

Internalin and Related Proteins: A Family of Listeria monocytogenes Determinants that Mediate Entry into Nonphagocytic Cells

Listeria monocytogenes is a food-borne pathogen that, although most often not life-threatening, can occasionally cause serious illness in immunosuppressed individuals, pregnant women, and neonates. To cause disease, *Listeria* must traverse a series of formidable host barriers such as the intestinal epithelium and the placental and blood-brain barriers. It is thought that the ability of these bacteria to invade non-phagocytic cells plays an essential role in breaching those natural barriers. Efforts to identify *L. monocytogenes* determinants that mediate internalization resulted in the characterization of two related proteins, InlA, also known as internalin, and InlB (Cossart and Lecuit, 1998). InlA is required for *L. monocytogenes* entry into the enterocyte-like intestinal epithelial cell Caco-2, while InlB mediates entry into hepatocytes and several endothelial, epithelial, and fibroblast-like cell lines. Both these proteins are able to mediate the internalization of coated latex beads, indicating that they are not only necessary, but also sufficient to trigger bacterial internalization. InlA and InlB share significant amino acid sequence similarity and belong to a *Listeria* protein family with at least five additional members. A common feature of this protein family is the presence at their amino terminus of a domain composed of a varying number of leucine-rich repeats (LRRs). This domain is critical for the stimulation of bacterial entry. It has been proposed that the composition and number of the LRRs establishes the receptor specificity of these bacterial ligands which, in turn, determines their individual contribution to bacterial entry into different mammalian cells. Recently, the x-ray crystal structure of the leucine-rich repeats domain of InlB was solved, revealing intriguing features (Marino et al., 1999). The LRRs are arranged in tandem, giving the molecule an elongated and curved shape. Each repeat is composed of a β strand that alternates with an opposing antiparallel helix, and the repeats are connected by coils. The β strands are highly conserved but the helices are divergent, exhibiting different register and type. Of note is the presence of an amino terminal ~40 amino acid domain with two highly solvent-exposed calcium ions. These calcium ions do not serve structural purposes; therefore, it has been proposed that they may be involved in InlB-receptor binding. This hypothesis is supported by the observation that the calcium ions are not only highly exposed but also incompletely coordinated, leaving as many as five oxygen atoms available for interaction with other proteins. Although intriguing, so far there is no evidence to support this interesting model.

Signaling for Entry: PI-3 Kinase and Beyond

The signaling events leading to InlA- and InlB-mediated entry have been partially characterized (Ireton et al., 1996, 1999). The actin cytoskeleton, tyrosine phosphorylation, and phosphoinositide (PI) 3-kinase activity are required for entry through both these pathways. Infection of cells with *L. monocytogenes* as well as treatment with purified InlB leads to the activation of the PI 3-kinase (p85-p110), the tyrosine phosphorylation of the adaptor proteins Gab1, Cbl, and Shc, and the formation of a complex between p85-p110 and Gab1. These events are predicted to trigger further downstream signaling that eventually leads to bacterial uptake. The nature of these signaling events is not known. However, they may involve the activation of actin-organizing, small GTP binding proteins such as Rac1, as this GTPase has

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been shown to act downstream of p85-p110 to mediate actin cytoskeleton rearrangements in response to certain stimuli in some cell types.

E-Cadherin, the Receptor for InlA

An affinity chromatography strategy identified E-cadherin as the receptor for InlA (Mengaud et al., 1996). E-cadherin is a calcium-dependent, cell adhesion molecule that plays an essential role in the formation of intercellular junctions, epithelial cell polarization, and general maintenance of tissue architecture. E-cadherin is composed of an extracellular region made of five repeated ectodomains, a transmembrane segment, and an intracytoplasmic domain that interacts with a group of cytoskeleton-associated proteins called catenins. A series of mutagenesis and domain swapping experiments have mapped a residue (a proline at position 16) that is critical for InlA binding (Lecuit et al., 1999). Interestingly, mouse E-cadherin has a substitution at this position and therefore InlA does not promote bacterial entry into mouse cells. This finding may explain the observation that InlA is dispensable for mouse virulence and might also provide the basis for establishing an animal model to assess the role of InlA in virulence.

The mechanisms by which the internalin/E-cadherin interaction leads to bacterial uptake are not well understood. Signaling through this receptor to promote actin cytoskeleton reorganization to drive bacterial internalization is most likely essential since inhibitors of tyrosine kinases and PI 3-kinase blocked InlA-mediated entry (Ireton et al., 1996). Disruption of the interaction of E-cadherin with α- and β-catenins effectively prevented InlA-mediated bacterial entry without disrupting bacterial binding to the receptor (Lecuit et al., 2000). These results indicate that the direct association of the receptor with the actin cytoskeleton is essential for mediating entry. Whether such association is required to transduce downstream signaling events or simply to facilitate the progressive apposition of the plasma membrane around the incoming bacteria is not known. There is an interesting parallel between InlA-mediated entry and invasin-mediated entry. Invasin is an unrelated outer membrane protein of the enteropathogenic bacteria *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* that promotes entry by binding to a different set of cell adhesion molecules, the β_1 integrin family of proteins (Isberg et al., 2000). Like InlA-E-cadherin, invasin-mediated signaling through β_1 integrins is required for entry. However, in contrast to E-cadherin, mutations that interfere with the association of β_1 integrins to the actin cytoskeleton enhance rather than prevent invasin-mediated uptake. Most likely these mutations facilitate the lateral movement and the subsequent recruitment and clustering of the integrin receptors that are necessary to stimulate signaling. Invasin-mediated signaling alone, however, is not sufficient to promote bacterial uptake. In addition, high affinity binding of invasin to multiple integrin receptors is necessary for the bacterial surface to serve as a template for the complete "zippering" of the cell plasma membrane about the bacterium. Whether the binding of InlA to the ectodomain of E-cadherin serves a similar purpose in *L. monocytogenes* entry is not known.

Multiple Receptors for InlB

Two recent papers, one of them in this issue of Cell (Shen et al., 2000), report the identification of different receptors for InlB. Braun et al. used affinity chromatography to isolate InlB-interacting proteins (Braun et al., 2000). This approach led to the identification of gC1q-R, the receptor for the globular head of the complement component C1q. In addition to C1q, this receptor has been reported to bind multiple ligands such as thrombin, vitronectin, and the HIV-transactivator Tat. The significance of the promiscuity of binding of this receptor is not clear. The binding of InlB to gC1q-R is strictly dependent on the presence of divalent cations. However, it is not known whether this binding is mediated by the highly exposed calcium ions observed in the crystal structure of the InlB LRRs internalization domain. gC1q-R is ubiquitously expressed in most tissues and cells, which would be consistent with the observation that InlB mediates entry into a broad range of cells. The involvement of gC1q-R in InlB-mediated entry is supported by the observation that antibodies to the gC1q-R or addition of its ligand, C1q, were able to inhibit InlB-mediated entry. Furthermore, a cell line expressing apparently nonfunctional low levels of gC1q-R and therefore refractory to InlB-mediated entry was rendered susceptible by transiently expressing high levels of gC1q-R. The identification of gC1q-R as the InlB receptor was surprising, as this receptor does not possess a transmembrane domain or a consensus site for glycosylphosphatylinositol membrane anchoring. Thus, the mechanism by which gC1q-R associates with the cell membrane and subsequently signals for internalization remains unclear. The possibility of the requirement of a coreceptor for its signaling function has been proposed.

The identification of the second receptor for InlB resulted from the characterization of proteins that become tyrosine phosphorylated after treatment of cells with soluble InlB (Shen et al., 2000). Soluble InlB is able to stimulate signaling events similar to those promoted by *Listeria*, including PI-3 kinase activation and protein tyrosine phosphorylation. An $\sim$145 kDa protein that became tyrosine phosphorylated upon treatment of cells with soluble InlB was identified and shown to correspond to the Met tyrosine kinase (Met). Met is a high-affinity receptor for hepatocyte growth factor (also known as scatter factor). Several properties of Met make it a logical candidate to be a receptor for InlB. These include its coupling to p85-p110 activation, its association with the adaptor proteins Gab1 and Cbl, and its distribution in tissues and cells where InlB is thought to exert its function. The involvement of Met as the InlB receptor is directly supported by several pieces of evidence: (1) InlB induces responses similar to those induced by HGF, the natural ligand of Met, such as the tyrosine phosphorylation of Met, Gab1, and Cbl and the stimulation of cell scattering; (2) InlB directly binds to Met through its LRR domain; (3) a cell line expressing low levels of Met and unable to support InlB-mediated bacterial uptake was rendered permissive for entry by the expression of high levels of Met; and (4) antibodies directed to Met or a soluble form of the receptor inhibit bacterial entry.

The identification of two different receptors for the InlB protein raises the question whether there is a functional relationship between Met and gC1q-R or whether these receptors work independently. There is no evidence to support or rule out a functional interaction

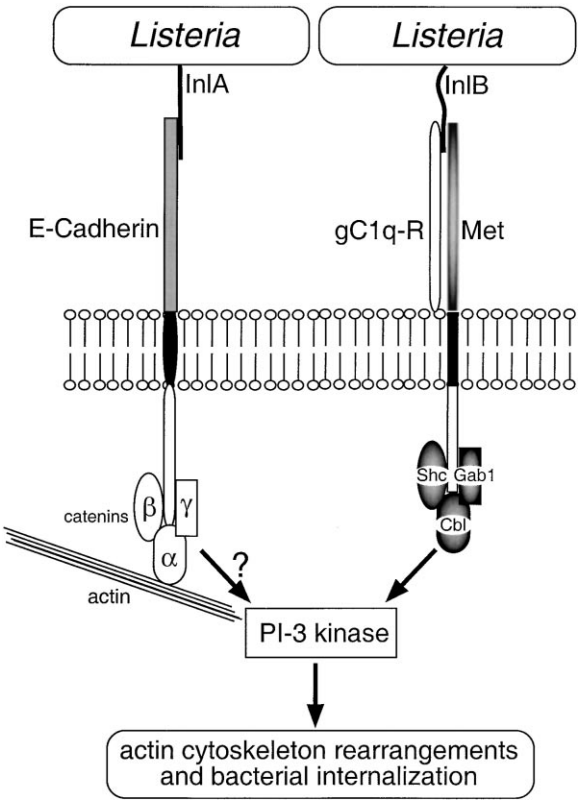

Figure 1. Hypothetical Diagram of the Alternative Pathways for *Listeria monocytogenes* Entry into Cells

As described in the text, it is not yet clear whether gC1qR and c-Met act as coreceptors or independently.

between these two receptors. However, the lack of a transmembrane domain in gC1q-R predicts the requirement of a coreceptor for transmembrane signaling. Therefore, it is possible that such a coreceptor may be Met. An answer to this question would be facilitated by the availability of a cell line completely devoid of both receptors. Nevertheless, a good start may be the examination of the expression of Met and gC1q-R in the gC1q-R- and Met-defective cell lines used in these studies. This type of analysis could determine whether either of these receptors is sufficient to support InlB-mediated uptake.

It is also possible that InlB can bind these receptors simultaneously through different domains. The calcium dependence of InlB binding argues that binding to gC1q-R may occur through the exposed calcium ions, as suggested by the x-ray crystal structure of the InlB LRR domain. On the other hand, there is no evidence that InlB binding to Met requires calcium, arguing that InlB binds this receptor through a domain distinct for the calcium binding region. The fact that Braun et al. did not identify Met in their affinity purification of InlB binding proteins suggests that cations are not required for InlB binding to Met, as the elution of the bound proteins in their affinity experiments was carried out by the addition of EDTA (Braun et al., 2000). This approach obviously favors the identification of ligands that require divalent cations for binding. A hypothetical diagram of the signaling pathway is shown in Figure 1.

Multiple Bacterial Ligands and Cellular Receptors to Breach Different Host Barriers?

Evidence indicates that, in vitro, InlA and InlB mediate entry into different cells, and therefore, it has been proposed that these bacterial ligands play different roles during bacterial infection. Mouse infection studies have suggested that InlB plays an important role in the infection of hepatocytes (Gaillard et al., 1996). In addition, the tissue distribution of its receptor Met suggests the possibility that InlB may also be involved in the ability of *L. monocytogenes* to breach the placental barrier and infect the fetus. Met is expressed in the trophoblast of the placenta and *L. monocytogenes* has been observed to infect the trophoblast of pregnant mice. It is therefore possible that the ability of *L. monocytogenes* to breach the placental barrier and fetal endothelial cells is mediated by InlB/Met interactions.

Investigation of the contribution of InlA to bacterial infection has been hampered by a lack of a suitable animal model as mouse E-cadherin does not support InlA-mediated entry (Lecuit et al., 1999). It has been hypothesized that InlA mediates entry into intestinal epithelial cells since, at least in vitro, this protein mediates entry into the intestinal epithelial cell line Caco-2. However, E-cadherin is only expressed on the basolateral side of polarized cells and therefore would not be available for interaction with its bacterial ligand when *L. monocytogenes* is in the intestinal lumen. It is possible that *L. monocytogenes* breaches the intestinal barrier through the more permissive M-cells that line the intestinal epithelium and subsequently gains access to the intestinal epithelium through the basolateral side where E-cadherin is expressed. Alternatively, *L. monocytogenes* may induce signaling events that may lead to a transient depolarization of the intestinal epithelium, resulting in the availability of E-cadherin for interaction with InlA. E-cadherin is also expressed in the choroid plexus and therefore it is possible that InlA helps *L. monocytogenes* to breach the blood-brain barrier. A more thorough examination of the tissue distribution of the different receptors for InlA and InlB will be required to obtain a coherent picture of the role of the different receptors and bacterial ligands in the pathogenesis of *L. monocytogenes* infection. In addition, the investigation of the potential role of other internalin-like molecules in *L. monocytogenes* pathogenesis has the potential to reveal additional pathways of bacterial entry. The study of this versatile family of bacterial ligands promises to uncover not only novel therapeutic avenues but also a better understanding of the function of important cellular receptors.

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